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Published in:
Aquaculture

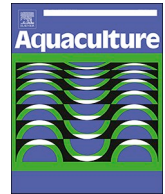
DOI:
[10.1016/j.aquaculture.2020.735268](https://doi.org/10.1016/j.aquaculture.2020.735268)

Publication date:
2020

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Fossmark, R. O., Vadstein, O., Rosten, T. W., Bakke, I., Koseto, D., Bugten, A. V., Helberg, G. A., Nesje, J., Jorgensen, N. O. G., Raspati, G., Azrague, K., Østerhus, S. W., & Attramadal, K. J. K. (2020). Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*). *Aquaculture*, 524, [735268]. <https://doi.org/10.1016/j.aquaculture.2020.735268>



Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*)

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ARTICLE INFO

Keywords:

Recirculating aquaculture systems (RAS)
Microbial matured water
Membrane filtration
Organic loading
Atlantic salmon

ABSTRACT

A key challenge in recirculating aquaculture systems (RAS) is the accumulation of particulate organic matter, especially the fine and colloidal fraction due to low removal efficiency of today's technology. The supply of organic matter is typically the limiting resource determining the carrying capacity (CC) of heterotrophic bacteria in the system. An appropriate and stable CC is proposed as a strategy for an optimal microbial environment in RAS with less blooms of opportunistic bacteria and more stable community dynamics. In this study, we investigated the effects of including a membrane for ultrafiltration in the RAS water treatment loop (treating 10–15% of the total water flow) to reduce the amount of fine and colloidal organic matter. Atlantic salmon parr (*Salmo salar*) were reared in two pilot-scale RAS (mRAS: membrane, cRAS: conventional). To evaluate the bacterial dynamics with and without membrane filtration at different organic loadings, the water exchange rates of the systems were manipulated equally to create periods with high and low loading of organic matter. The results showed that in the mRAS water, the level of organic matter was more stable throughout the experiment for the changing organic matter loadings. As a consequence, water in mRAS had higher microbial diversity, lower and shorter bacterial blooms and generally lower bacterial densities than in cRAS. All variables indicate a better microbial environment in the water of the system with membrane filtration. Also, the physicochemical water quality was better in mRAS in terms of lower turbidity and particulate organic matter (POC), and slightly lower concentrations of total ammonia nitrogen (TAN). The composition of the microbial communities was significantly different between the two systems, and temporal variations in the community dynamics were observed in both systems during the periods with different organic loadings. At high organic loading, the genus *Mycobacterium* had high relative abundance in the cRAS water (up to 0.25) compared to mRAS (0.01–0.03). The fish in mRAS were significantly bigger (14%) than fish in cRAS at the end of the experiment, however it is hard to conclude whether the better growth in mRAS was due to higher temperatures (caused by membrane operation) or better water quality, as it was probably a combination of both. We can conclude that membrane filtration gave more stable and better physicochemical and microbial water quality, which will reduce the probability for microbially related accidents in RAS.

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1. Introduction

Optimization of water treatment to obtain and maintain a healthy bacterial flora in recirculating aquaculture systems (RAS) is gaining interest (Attramadal et al., 2012; Wold et al., 2014; Pedersen et al., 2017; Rud et al., 2017; Rojas-Tirado et al., 2018; de Jesus Gregersen et al., 2019). Bacteria are necessary for the fluxes and the conversions of nutrients in RAS to maintain high water quality (Blancheton et al., 2013). Bacteria also have direct implications for the fish, as they are highly abundant in the water and in constant contact with the mucosal surfaces of the skin, gills and gut. This close relationship can be both beneficial and detrimental for the fish. Bacteria can give positive effects through metabolic and immunological relations, such as improved utilization of nutrients in the gut and protection against invasion of pathogens (Nayak, 2010; Maynard et al., 2012; Gomez et al., 2013). On the downside, negative interactions with bacteria challenge the fish and can lead to infections (Llewellyn et al., 2014). The microbial community structures in RAS are shaped by physicochemical variables and competition for nutrients and space, and this selection has consequences for the composition of the microbial communities (De Schryver and Vadstein, 2014).

Rearing regimes selecting for mature microbial communities in the water, including RAS, have been shown to be beneficial for the cultivation of marine fish larvae (Skjermo et al., 1997; Attramadal et al., 2014; Attramadal et al., 2016; Vestrum et al., 2018; Vadstein et al., 2018a). RAS have properties that favour the development of matured communities dominated by K-strategic bacteria, which are considered to be beneficial for the fish (Attramadal et al., 2012; Attramadal et al., 2014). K-selected microbial communities can outcompete opportunistic r-strategic bacteria and lower the chance of negative bacterial interactions with the fish (Vadstein et al., 2018b). However, systems for juvenile and on-growing salmon have not been studied in this context. There is limited knowledge of the effects of bacterial communities on salmon fish health and growth. Systems for salmon parr and smolt production have lower salinity, and substantially higher biomass, organic load and higher water exchange rate in the fish tanks than the, in this context, more studied systems for marine larvae. These differences between systems are expected to affect the microbial dynamics significantly.

The availability of nutrients is a key to the selection regime for microbially matured water. The removal of particulate organic carbon (POC) originating from feed waste and faeces in RAS is therefore essential to consider, as POC can mineralize and dissolve to become bacterial substrate. Biodegradable organic matter is the limiting substrate for heterotrophic bacteria in RAS (Blancheton et al., 2013), thus the accumulation and solubilization of degradable POC increases the bacterial carrying capacity (CC) of the system. This in turn, increases bacterial numbers and can alter the microbial community composition due to changes in the selection pressure in the RAS (Attramadal et al., 2012; Wold et al., 2014). Efficient removal of POC is also important for maintaining high physicochemical water quality (Chen et al., 1993; Cripps and Bergheim, 2000; Michaud et al., 2006; Fernandes et al., 2015). However, the large variety of particle sizes in RAS (diameters of nanometres to centimetres), challenges the choice of water treatment technology. The conventional particle removal systems used in freshwater RAS cannot efficiently remove particles smaller than 20 µm (Chen et al., 1993; Chiam and Sarbatly, 2011). Protein skimmers have shown to be efficient for fine particle removal in seawater systems (Rahman et al., 2012), however they are not as efficient in freshwater. Smaller particles may therefore accumulate and negatively affect the water quality by increasing the CC, and thus reduce the nitrification efficiency of the biofilter (Chen et al., 2006; Michaud et al., 2006; Guerdat et al., 2011), irritate the gills of the fish (Chapman et al., 1987), shield bacteria from disinfection (Hess-Erga et al., 2008) and result in formation of anaerobic zones which can yield production of toxic hydrogen sulphide (H₂S) (Tal et al., 2009).

Membrane technology with ultrafiltration (0.001–0.1 µm) or microfiltration (0.1–10 µm) has been proposed as supplement to existing particle removal technologies, especially targeting the fraction of fine and colloidal particle sizes to improve water quality in RAS (Gemende et al., 2008; Pimentel et al., 2017). By reducing the nutrient supply per bacterium, a competitive environment favouring K-selection of the bacteria in the water may be achieved (Attramadal et al., 2014). Membrane filtration have previously showed increased growth rates and reduced mortality in cultivation of marine fish larvae due to improved water quality (Holan et al., 2014; Wold et al., 2014). Our hypothesis is that RAS in general selects for microbial matured water of high stability, and that membrane filtration in RAS increases the stability even more by keeping the CC lower and more even, which will be more critical in a system with high biomass and high organic loading.

Membrane filtration in RAS with juvenile and on-growing salmon is not well studied, and we lack knowledge on whether microbially matured water could be achieved in RAS with fresh water, high biomass and low hydraulic retention time in the rearing tanks. Membrane filtration adds additional costs and complexity to the RAS for the fish farmers, and it demands frequent cleaning due to fouling (Viadero and Noblet, 2002; Sharrer et al., 2007). More research is therefore needed to evaluate if the cost of membrane filtration for improved particle removal can be balanced by better physicochemical and microbial water quality yielding healthier salmonids. The objective of this study was to evaluate how the physicochemical and microbial water quality of RAS with and without membrane filtration were affected by high/increasing and low/decreasing loads of particles and organic matter. We hypothesized that the water in RAS with improved particle removal by membrane filtration would 1) result in lower organic loads, lower numbers of bacteria and a more stable and favourable microbially matured water, and 2) better general physicochemical water quality and nitrification efficiency in the biofilter, all of which could contribute to improved growth and survival of the fish.

2. Materials and methods

2.1. Experimental design: system configuration and rearing conditions

The experiment was conducted at Sealab, NTNU's Centre of Fisheries and Aquaculture in Trondheim, Norway. It included two separate pilot-scale RAS, the conventional RAS (cRAS) and the RAS with a membrane (mRAS), each with a total volume of 4.2 m³ (Fig. 1). The water in the systems were from the municipal freshwater distribution system. Some seawater (pumped in from 70 m sea level depth in the Trondheim fjord and UV-treated) was mixed into the systems to maintain 3 ppt salinity to avoid growth of fungus. Both systems included six rearing tanks (0.40 m³ each) and a recirculating water treatment loop containing a mechanical drum screen filter (HEX, CM Aqua Technologies, Denmark), a moving bed biofilm reactor (MBBR: Nofitech, Norway) and a water-to-air counter flow CO₂-degasser (Nofitech, Norway). The MBBR included three chambers, each filled with 0.12 m³ of carriers with a specific surface area of 828 m² m⁻³ (Table 1). The membrane in mRAS was a hollow fibre, polymeric X-FLOW Compact 4.0G ultrafiltration membrane (Pentair, Netherlands). Ultrafiltration was chosen over microfiltration due to less chances of irreversible fouling (Kimura et al., 2006). The module contained two membranes in series, each with an area of 4.0 m² and an average pore size of 20–30 nm. The membrane treated 10–15% of the total water flow and was backwashed every 60 min (timer controlled) for 50 days, then every 15 min for the rest of the experiment (22 days). Chemical cleaning with sodium hydroxide (NaOH) and sodium hypochlorite (NaOCl) was done every 2nd week manually. The membrane was operated with constant transmembrane pressure (TMP) at 0.7–0.8 bar, with a crossflow configuration and produced 600–700 L h⁻¹ permeate. The membrane maintained 98.5% recovery from feed stream on average through the experiment.

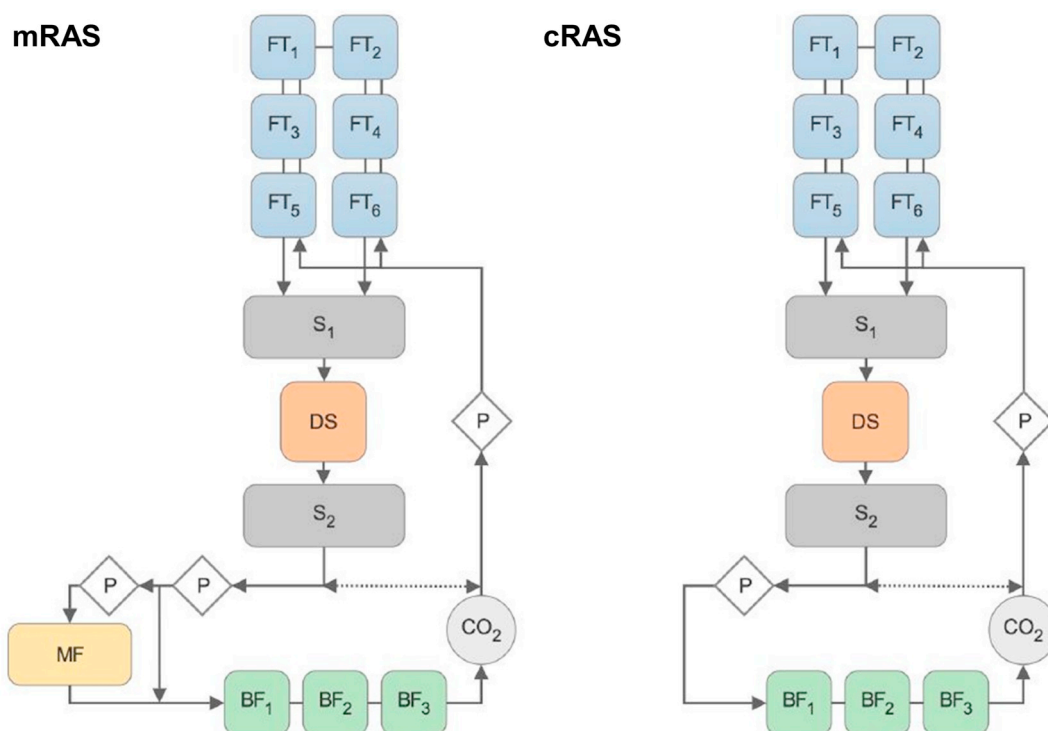


Fig. 1. Experimental set-up of the two systems, membrane RAS (mRAS) and conventional RAS (cRAS). FT = fish tank, S = sump, DS = drum screen filter, MF = membrane filtration, BF = biofilter, CO₂ = CO₂-degasser, P = pump.

Table 1
Dimensions of components in mRAS and cRAS.

Location	Parameter	Unit	
Total system	Volume	m ³	4.2
	Make-up water flow	L h ⁻¹	Table 2
	HRT (volume (make-up water flow ⁻¹))	days	Table 2
Culture tanks	Volume	m ³	0.40
	Flow	L min ⁻¹	17
	HRT (volume (flow ⁻¹))	min	23
Drum screen filter	Mesh	μm	mRAS: 20 cRAS: 60
CO ₂ degasser	Area	m ²	6.85
MBBR (3 chambers)	Reactor volume empty	m ³	0.40 × 3 = 1.2
	Volume carriers	m ³	0.12 × 3 = 0.36
	Carrier specific surface	m ² m ⁻³	828
	Biofilter carrier area	m ²	99 × 3 = 298
	Filling fraction (dry)	% of volume	30

Each fish tank was stocked with 60 individuals (6 kg m⁻³) of Atlantic salmon parr (*Salmo salar*) with an average weight of 40 ± 4 g (± SD). After stocking, the systems were run for an acclimatization period of 70 days where the water was cross run between the systems to ensure an equal start situation. The fish came from MOWI Slørdal and had been reared in a flow-through system before arriving at Sealab. The fish were reared with an artificial winter light regime (7 L: 17D) throughout the experiment. They were fed commercial pellets (3 mm Nutra RC, Skretting, Norway) by automatic feeders (Arvo-Tec Oy, Finland) installed at each fish tank. The fish were fed every 20 min during the 7-h light period.

The experiment, excluding the 70-day acclimatization period, lasted for 72 days in total (03.04.17–13.06.17). By manipulation of the make-up water exchange rate/total hydraulic retention time (HRT) and feeding, we divided the experiment into three different periods (Table 2): Period 1 (P1) got an increasing load of organic matter

(accumulating) through the period due to low water exchange rate, P2 got decreasing organic loading due to high water exchange rate (dilution), and P3 got increasing organic loading (accumulating) due to lower water exchange rate and higher feeding. At the end of P1 there was extra addition of water to both systems as one of the outlets of a fish tank in cRAS clogged, and water ran out of the tank and was lost. New water was added to both systems at day 12 to 18 to compensate for the loss in cRAS, and to assure identical conditions in both systems (not included in Table 2).

2.2. Daily management and water quality variables

The fish were inspected on a daily basis, and any mortality/abnormalities were documented. The experiment was carried out within the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th June 2009, at a facility with permission to conduct experiments on fish (code 93) provided by the Norwegian Animal Research Authority (NARA) by FELASA-approved personnel. Large particles of feed waste and faeces were collected on sieves in the outlet of each fish tank and removed manually daily. Temperature and dissolved oxygen were measured with a handheld Pro2030 dissolved oxygen meter (YSI, USA) in the outlet of the tanks. The concentration of CO₂ was measured (Oxyguard, Denmark) in the pump sump after the CO₂-degasser. Total ammonia nitrogen (TAN), nitrite and nitrate were analysed in water entering the biofilter with a DR/890 Colorimeter (Hach, USA). The pH was recorded with a 3210 pH-meter (WTW™ ProLine™, Xylem, Germany), and alkalinity was measured through acid titration of RAS water with 0.1 M hydrochloric acid (HCl) until the titration endpoint of pH 4.5. Sodium bicarbonate (NaHCO₃) was added to the systems to maintain an alkalinity of 50 mg L⁻¹ CaCO₃ throughout the experiment. All these measurements were performed either daily or every other day. The turbidity was measured in glass vials using a 2100AN turbidimeter (Hach, USA) twice a week. Samples for total and dissolved organic carbon (TOC and DOC) were collected every 2 weeks in glass vials and

Table 2

Experimental conditions in mRAS and cRAS for the three periods of different organic loading.

Day	Period	Water exchange rate (% d ⁻¹)	HRT _{tot} (days)	Variation in organic matter load through period	Make-up water flow (L h ⁻¹)
1–18	P1	10	9.7	Increasing ↑	18
19–51	P2	60	1.6	Decreasing ↓	105
52–72	P3	30	3.5	Increasing ↑	50

conserved with acid until analysis through combustion and carbon dioxide detection (Apollo 9000 TOC-analyser, Teledyne Tekmar, USA). The water for DOC analysis was filtered through 0.45 µm nitrocellulose membrane filters (MF-Milipore™). The difference between TOC and DOC was considered to be particulate organic carbon (POC), and the percentage of the particulate fraction of TOC was calculated.

2.3. Water sampling for microbiology

Water samples for analysis of microbiology were collected at Day 1, 18, 39, 50, 66 and 72 in the outlet of fish tanks 1, 3 and 5, inside the DS filter, sump 2 and after biofiltration before CO₂-degassing (Fig. 1). The sampling was done at the same time of the day (around 10.00 AM). Water samples were filtered through a 0.22 µm sterile filter (Sterivex™) using a 60 mL syringe. The volume filtrated was approximately 200 mL for mRAS and 100–150 mL for cRAS, depending on amount of particles in the water. All the samples were stored at –20 °C until further analysis.

2.4. Sequencing of 16S rRNA gene amplicons

2.4.1. DNA extraction, PCR and Illumina sequencing preparation

DNA extraction was conducted with the QIAamp® DNA Mini Kit (Qiagen, Germany). The water filters were cut into small pieces with a sterile scalpel and put into 1.5 mL Eppendorf tubes. The manufacturers' protocol was followed with minor alterations. An extra lysis step was added to ensure lysis of Gram-positive bacteria by using an enzymatic lysis buffer (180 µL) consisting of 2 mM EDTA, 20 mM Tris-HCl (pH 8), 1.2% Triton and lysozyme (20 mg mL⁻¹), following 1 h of incubation at 37 °C. The variable region 4 (V4) of the bacterial 16S rRNA gene (Marchesi et al., 1998) was targeted for bacterial community analyses of RAS water and biofilter biofilm. The V4 region was amplified using the broad coverage primers 515F (5'-tcgtcggcagcgctcagatgtctataagacagcagcnnnn GTGCCAGCMGC GGTA-3'), and 805R (5'-gtctcgtgggctcggagatgtgtataagacagcnnnnACTACNVGGGTATCTAA KCC-3'). Illumina adapter sequences are in lower case letters and were included due to subsequent amplicon sequencing. Each PCR reaction contained 0.02 U/µL Phusion Hot Start II DNA polymerase (Thermo Scientific), 0.2 mM of each dNTP (VWR), 0.3 µM of each primer (SIGMA), 2 mM MgCl₂ (Thermo Scientific), and reaction buffer (Thermo Scientific) in a total reaction volume of 25 µL, including 1 µL of ~1 ng/µL DNA extract as template. The PCR reactions were run with 30 cycles (T100TM Thermal Cycler, BioRad). PCR products were normalized with a SequalPrep Normalization Plate (96) kit (Invitrogen, USA), following the protocol included in the kit. Unique barcode-sequences were added to each PCR product using the Nextera XT Index kit (Illumina, USA) through an additional PCR run with 8 cycles. The barcoded PCR products were examined by agarose gel electrophoresis. The indexed amplicons were normalized again using the Normalization plate. A total of 96 samples were pooled for each illumina lane and concentrated with AmiconUltra 0.5 Centrifugal Filter (Merck Millipore, Ireland) as described by the manufacturer. The concentration and purity (A260/280 & A260/230) of the sample were measured with NanoDrop One (Thermo Scientific). The pooled amplicon libraries (96 samples in each) were sequenced on one MiSeq lane each (Illumina, USA) at the Norwegian Sequencing Centre (NSC, Oslo, Norway).

2.4.2. Processing of sequencing data

The Illumina sequencing data were processed with the USEARCH pipeline (version 9.2). Paired reads were merged, primer sequences trimmed and reads shorter than 230 base pairs were filtered out. The data went through demultiplexing and quality filtering with the Fastq_filter command with an expected error threshold of 1.0. Singletons and chimera sequences were removed, and clustering at the 97% similarity level was done, all with the UPARSE-OTU algorithm (Edgar, 2013). Finally, taxonomy assignment was performed using the Syntax script (Edgar, 2016) with the RDP reference data set (version 15) and a confidence value threshold of 0.8. The data were normalized to lowest read count (20,000) to avoid bias in diversity analyses due to variable sequencing depth. OTUs representing eukaryotic amplicons (algae) were removed before further statistical analysis. OTUs of interest were further investigated with the SeqMatch tool for type strains at the RDP website (Cole et al., 2013).

2.5. Quantification of bacterial cell densities and growth

2.5.1. Flow cytometry

Bacterial numbers were quantified by flow cytometry of water from fish tanks, sumps and the CO₂-degasser outlet three times a week. The samples were fixated with glutaraldehyde (final concentration 0.5%), snap frozen in liquid nitrogen and stored at –20 °C. Prior to analysis, the samples were diluted 1:10 with 0.2 µm filtered 0.1 × TE buffer to keep the cell counts below 1000 events µl⁻¹ for avoiding overload of the instrument's max read count. SYBR® Green I (Life Technologies, Thermo Fisher Scientific Inc.) DNA stain was added to the samples (final concentration 1%) to stain the bacteria. The samples were analysed with a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, USA). The stained cells were excited with a blue laser (488 nm). The detector for green fluorescence (533 ± 15 nm) was used to detect the bacteria present in the sample. Further analysis of the results was conducted using the BD CSampler™ Software, and bacterial densities were calculated.

2.5.2. [³H]-thymidine incorporation

Incorporation of [³H]-thymidine into bacterial DNA was performed according to Fuhrman and Azam (1980) to estimate bacterial cell growth. Into triplicates of water samples of 1 mL, [³H]-thymidine was added to a final concentration of 10 nM and total activity of 0.5 µCi (specific activity of 20 Ci mmol⁻¹) (PerkinElmer, USA). Incubation period was 30 min at fish tank water temperature with shaking at 200 RPM. The incubations were terminated by addition of 50% trichloroacetic acid (TCA) to a final concentration of 5%. As controls, 50% TCA was added to 0.22 µm-filtered fish tank water to a final concentration of 5% prior to [³H] thymidine additions and run in parallel to measure abiotic adsorption of radioactivity. Finally, the samples were centrifuged, and the pellets were washed twice in 5% TCA. After removal of the supernatant, the pellets were suspended in 1 mL HiSafe® 3 scintillation fluid (PerkinElmer, USA). Radioactivity was counted in a PerkinElmer Tri-Carb 4910TR scintillation counter. The incorporation rate was converted to bacterial growth rate using a conversion factor of 1.1 · 10¹⁸ cells per mol thymidine (Riemann et al., 1987).

2.6. Analysis and assimilation of dissolved free amino acids

Water samples of 10 mL for analysis of concentrations of dissolved free amino acids (DFAA) were filtered through 0.2 µm membrane filters and kept frozen at -20°C until analysis by HPLC and fluorescence detection. Analysis of the amino acids followed procedure of Jørgensen and Middelboe (2006). DFAA were derivatized with *o*-phthalaldehyde and *N*-isobutyl-L-cysteine as a chiral agent (Brückner et al., 1995) and separated on a Waters XTerra RP18 3.5-µm particle column (Waters Corporation, Milford, USA) at a flow rate of 0.7 mL min^{-1} . Mobile phases were (A) aqueous solution of 5 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 45 mM sodium acetate trihydrate and 7.5% methanol at pH 6.4, and (B) 100% methanol (Mopper and Furton, 1991). The derivatives were eluted with the following gradient: T_0 min (100% A, 0% B), T_{27} min (50% A, 50% B), T_{30} min (10% A, 90% B) and T_{33} min (100% A, 0% B). The HPLC equipment consisted of a Waters 2965 autosampler and pump module, and Waters 2475 fluorescence detector. For calibration, a standard mixture of 18 L amino acids was enriched with non-protein amino acids and selected D isomers of amino acids (Glu, Asp, Ser and Ala) and glucosamine (GluA, component of bacterial cells walls). Individual amino acids in the chromatograms were identified from retention times determined from the standard mixture.

Bacterial assimilation of DFAA was measured according to procedure by Jørgensen et al. (1993). A mixture of four [^{14}C] L-amino acids (Glu, Ser, Gly and Ala) in an equimolar composition at a total activity of 0.01 µCi (PerkinElmer, USA) was added to triplicate 10 mL water samples and a control with 2% formaldehyde. Addition of the tracers corresponded to about 5 nmol DFAA L^{-1} . The samples were incubated for 30 min at fish tank temperature after which the samples were fixed with formaldehyde (2% final concentration). The fixed samples were filtered through 0.2 µm membrane filters which were radio assayed by liquid scintillation counting. Respiration of the assimilated DFAA was not determined.

2.7. Measures of microbial diversity and statistical analyses

The program package PAST version 3.21 (Hammer et al., 2001) was used to calculate diversity indices and to perform statistical analyses. Alpha-diversity measures included estimated species richness Chao-1 (Chao, 1984) and evenness. Beta-diversity was calculated based on the presence/absence-based Sørensen-Dice similarity and the abundance-based Bray-Curtis similarity (Chao et al., 2006). Water quality variables were checked for normality with Shapiro-Wilk test (Shapiro and Wilk, 1965). Two sample paired *t*-tests were used on data that did follow a normal distribution, whereas Mann-Whitney-Wilcoxon-tests were used on samples that did not. Two-Sample *t*-tests were used to determine statistical significance between fish end-weights and estimated richness and evenness. Ordination by Principal Coordinate Analysis (PCoA) with normalized and square root transformed data based on Bray-Curtis similarities were used to visualize the similarities/dissimilarities and development of the bacterial communities in mRAS and cRAS. Similarity Percentage (SIMPER) with Bray-Curtis similarities was used for assessing which OTUs that were mostly responsible for the observed differences in community composition between groups of samples (sampling dates and RAS system). One-way permutational multivariate analysis of variance (PERMANOVA) with Bray-Curtis similarities was performed to test for statistically significant difference between different groups of samples (Anderson, 2017).

3. Results

3.1. Performance of recirculating systems and fish

There was a significant 1.2°C higher temperature on average ($p < .0001$) in mRAS (Table 3), due to production of heat by the membrane filtration system during operation. The turbidity of cRAS

Table 3

Water quality variables through the experiment in mRAS and cRAS.

Variable	mRAS	cRAS
Temperature ($^{\circ}\text{C}$)	13.7 ± 0.6^b	12.5 ± 0.4^b
CO_2 (mg L^{-1})	1.83 ± 0.9	1.34 ± 0.9
Salinity (ppt)	3.5 ± 0.2	3.6 ± 0.2
pH	7.75 ± 0.02	7.74 ± 0.01
Alkalinity (mg L^{-1} as CaCO_3)	50.1 ± 1.5	50.7 ± 0.8
TAN (mg L^{-1}) ^a	0.079 ± 0.03^b	0.092 ± 0.03^b
$\text{NO}_2 - \text{N}$ (mg L^{-1}) ^a	0.051 ± 0.1	0.055 ± 0.1
$\text{NO}_3 - \text{N}$ (mg L^{-1}) ^a	21.2 ± 1.3	18.8 ± 1.3
Turbidity (NTU)	1.18 ± 1.03^b	3.52 ± 2.91^b

^a measured in water entering the biofilter.

^b significant difference between systems.

was 3 times higher than in mRAS ($p < .0001$), and the water was also visually more turbid with a stronger brown colour. Concentrations of TAN were 16% higher in cRAS, and a paired *t*-test confirmed that the difference was statistically significant ($p = .04$). The mortality was very low in both systems ($< 1\%$) with only 3 dead fish in each system through the experiment. The final weights of the fish in mRAS were 14% higher than in cRAS and significant ($p < .0001$) with averages (\pm SD) of $142.6 \pm 28.4\text{ g}$ and $125.0 \pm 23.2\text{ g}$, respectively.

3.2. Concentration of organic matter

The concentration of TOC was significantly higher in cRAS for all sampling dates ($p < .01$) and correlated with the organic loading during the different periods (Fig. 2A). The difference in concentration was particularly large at the beginning of P2 ($2\times$ higher in cRAS, $p = .002$). The concentration of DOC was slightly higher in cRAS than mRAS in the beginning of P1 and the beginning of P2 (Day 27: Fig. 2B). The difference was significant ($p < .01$), but the concentration was only 0.5 mg L^{-1} higher in cRAS. No significant differences in DOC were found between the systems for the rest of P2 and in P3 ($p > .06$). In the beginning of P1, POC constituted around 25% of the total organic carbon in both systems. Through P2, the amount of particles increased in cRAS and the particulate fraction of the total organic carbon was 35 to 40% at day 27 and 36, whereas in mRAS it was 15 to 20%. In P3, the difference between the systems were even more profound, with around 7% particulate organic carbon in mRAS and 30% in cRAS. The membrane had a clear effect on the concentration of particulate organic carbon, whereas the effect was not as evident for the dissolved organic carbon.

3.3. Bacterial densities, cell production and DFAA assimilation in RAS water

The bacterial densities in the RAS water were determined from water in fish tanks, sumps and CO_2 -degasser outlet and were similar in the different compartments of each RAS system (Low SD in Fig. 3). The trend in the densities of bacteria was negatively correlated to the water exchange rate and positively correlated to the organic load in both systems. The densities of bacteria were significantly higher in cRAS for all sampling dates ($p < .0001$). Three bacterial blooms were observed in cRAS, one in each period of the experiment. In mRAS, on the other hand, it was only tendencies for small blooms. During P1 with increased organic load the bloom in mRAS increased the bacterial density by a factor of 3 and in cRAS a factor of 9. This resulted in a 4 times higher maximum bacterial density in cRAS. The extra water exchange at the end of P1 (shaded grey) resulted in a quick reduction in the density of bacteria in both systems. The bloom during P2 was profound for cRAS and was 2.3 times higher than in mRAS and lasted much longer. The bacterial densities in cRAS declined a factor 20 from day 25 to the end of P2 (day 50). This reduction in bacterial numbers happened much

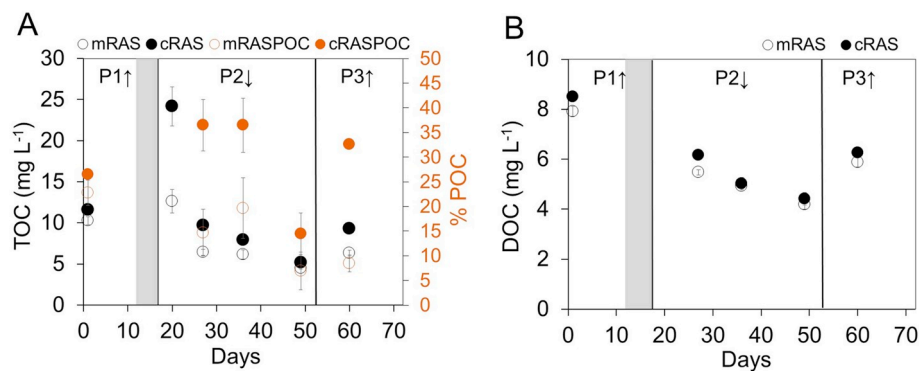


Fig. 2. A) TOC (black symbols) and %POC (brown symbols). B) DOC (black symbols) throughout the experiment. Data are the mean (\pm SD) from all samples in the same system (both sumps and CO₂-degasser outlet) each sampling day. Periods with different organic loading showed. Grey shading (day 12–18) shows extra addition of water due to technical problems.

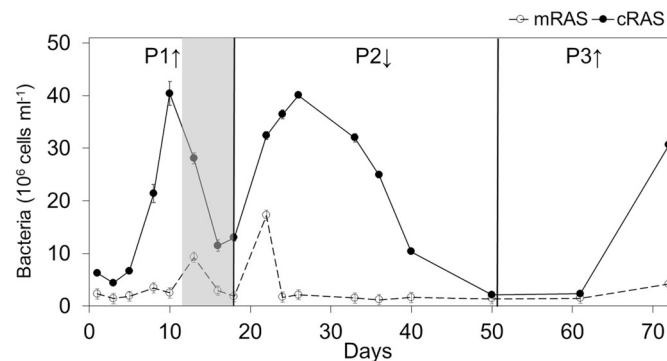


Fig. 3. Bacterial densities in RAS water through the experiment. Data are the mean (\pm SD) from all samples in the same system (fish tanks, sumps and CO₂-degasser outlet) each sampling day. Grey shading (day 12–18) shows extra addition of new water due to technical problems.

quicker in mRAS where it declined a factor 10 in only 2 days. During the overfeeding in P3 the bacterial densities increased by a factor 3 and 13 for mRAS and cRAS, respectively. At the end of the experiment, the density of bacteria was 7 times higher in cRAS than in mRAS. It is clear that membrane filtration reduced bacterial numbers.

The bacterial production showed minor variations in mRAS and varied significantly in cRAS during the experiment. In mRAS, the production ranged from 36 to 65 $\cdot 10^6$ cells L⁻¹ h⁻¹, while in cRAS the range was 27 to 915 $\cdot 10^6$ cells L⁻¹ h⁻¹ (Fig. 4A). On three out of five sampling days (Day 1, 18 and 72) the cell production was 3.1, 16.4 and 2.2 times higher in cRAS than in mRAS and was slightly lower on the other two days. The high bacterial production at Day 18 agrees with a high increase in cell density in cRAS the following days. The bacterial assimilation of DFAA (Fig. 4B) was less variable in mRAS than in cRAS and showed the same trend as observed for the cell production. In mRAS, the assimilation ranged from 0.77 to 2.19 μ g L⁻¹ h⁻¹, as compared to 1.1 to 13.3 μ g L⁻¹ h⁻¹ in cRAS. The assimilation was 2.7 to 6.1 higher in cRAS than in mRAS, except for Day 50 when the

assimilation was 2 \times higher in mRAS. When relating assimilation of C from DFAA to C required for the measured cell production, DFAA-C was determined to sustain from 34.5 to 90.9% of the microbial C demand in mRAS and 23.5 to 80.7% in cRAS. The lowest C contribution from DFAA in cRAS occurred when the peak in cell production occurred, suggesting that DFAA were not a major C source to the cells during that period.

3.4. Microbial diversity of system water

Illumina sequencing yielded a sequencing depth of on average 80,000 reads per sample, and in 400–800 observed OTUs per sample. Before further analysis the dataset was normalized to equal sequencing depth per sample (20,000 reads), to avoid bias in the further analysis.

3.4.1. Alpha diversity of water microbiota

Estimated species richness (Chao-1) for the water samples (Fig. 5A) was significantly higher in mRAS at all samplings in P2 and P3 ($p < .001$). The difference was especially evident at Day 39, which was approximately three weeks after the systems had gone from high to low organic loading. At this sampling, estimated richness in mRAS was approximately twice that in cRAS ($p < .0001$). The evenness (Fig. 5B) of the water microbiota was higher for mRAS than cRAS during P2 and P3 (Day 39, 66 and 72; $p < .001$). The difference was especially clear at Day 39 and Day 72 when evenness was about 2 \times higher in mRAS than in cRAS.

3.4.2. Beta diversity of water microbiota

To examine the temporal development within and between systems of the microbial communities, we used ordination by Principal Coordinate Analysis (PCoA) based on similarity indices. Bray-Curtis and Sørensen-Dice similarities were used to quantify whether differences in microbial communities were due to differences in relative abundance or change in OTU inventory. The first two coordinates (axes) of the ordination based on Bray-Curtis similarity explained 30.1 and 16.0% of the variation in community composition between samples, respectively

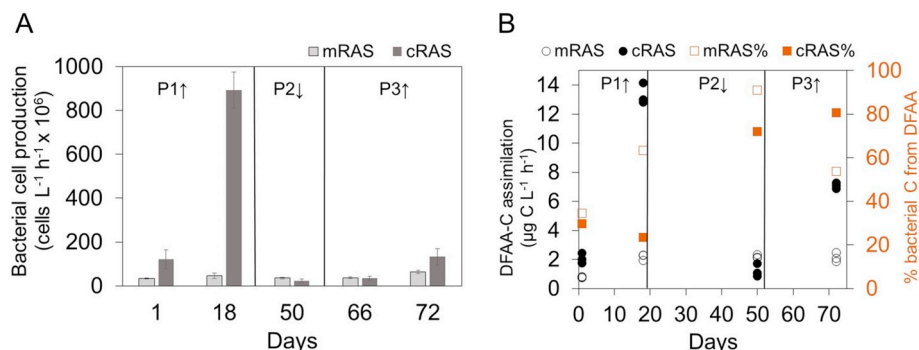


Fig. 4. A) Bacterial cell production. Data are the mean (\pm SD) from all samples in the same system (both sumps and CO₂-degasser outlet) each sampling day. B) Net incorporation of dissolved free amino acids (DFAA) into bacterial biomass. Contribution of DFAA-C to the bacterial C demand (%) is shown on the right (brown) y-axis in B.

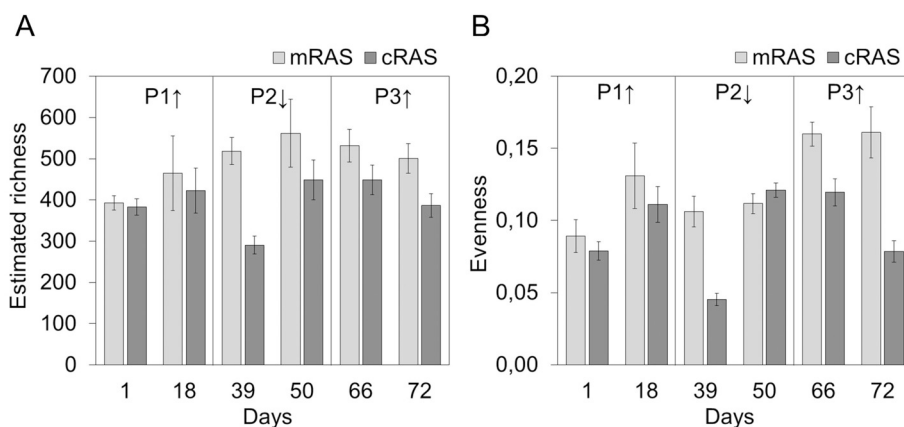


Fig. 5. A) Estimated richness (Chao-1) and B) Evenness for water samples through the experiment. Data are mean \pm SD of all samples in the same system (fish tanks, sumps and CO₂-degasser outlet) at each sampling date.

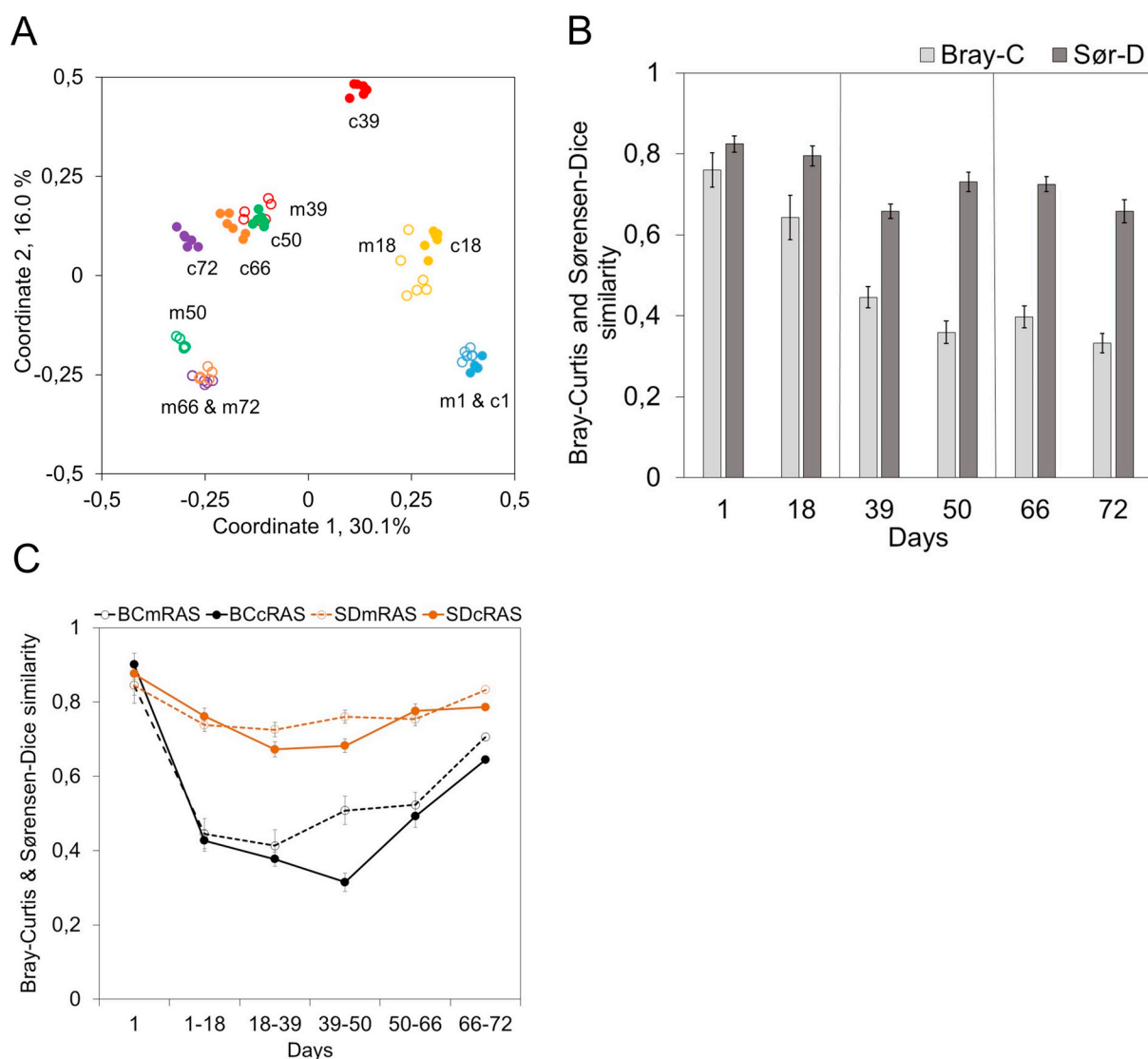


Fig. 6. A) PCoA of water samples based on Bray-Curtis similarities. m = mRAS and empty circles, c = cRAS and filled circles, following day of sampling (same colour). B) Bray-Curtis and Sørensen-Dice similarities between mRAS and cRAS. Data are mean \pm SD of all values in the similarity-matrix comparing all samples within each sampling date. C) Succession of microbial communities through the experiment within each system analysed with Bray-Curtis (black symbols/lines) and Sørensen-Dice (brown symbols/lines) similarities. Data are the mean \pm SD of all values in the similarity-matrix comparing water microbiota between two succeeding sampling dates within mRAS and cRAS.

(sum 46.1%: Fig. 6A). The PCoA plot shows that through P1, with increasing organic loading (Day 1 to 18), the communities of both systems developed in the same direction. The microbial community composition of the two systems were relatively similar on Day 18 following the first bacterial bloom (Fig. 3). In P2 on Day 39 (decreasing organic load), the communities of the two systems showed different succession pattern and continued to diverge until the end of the experiment. The samples from mRAS at Day 66 and 72 overlap, which is not the case for cRAS in P3. This indicates higher stability in mRAS than cRAS at the end of this period with increasing organic load. When comparing the two systems, the Sørensen-Dice similarity was higher than the Bray-Curtis similarity at all sampling dates (Fig. 6B). This indicates that variations in the relative abundances of the OTUs contributed more to the observed differences between the systems than the presence or absence of OTUs. The Bray-Curtis similarity decreased by a factor of 2 throughout the experiment, indicating that the cRAS and mRAS water microbiota developed to become increasingly dissimilar. Once the water microbiota of the systems diverged after Day 18 (Fig. 6A & B), the Bray-Curtis similarities decreased with 40% by Day 50 and stayed at the low similarity throughout the rest of the experiment (Fig. 6B). Thus, when the environmental shift (change in organic loading) induced the change in the microbial communities, they never returned to become more similar to each other again. In the succession plot within each system (Fig. 6C), the Sørensen-Dice is generally higher than Bray-Curtis similarities for all sampling dates. This shows that the change in abundance of OTUs present within each RAS contributed more to the succession than contribution from new OTUs. Bray-Curtis was only notably higher in mRAS from Day 39 to 50 with values of 0.3 and 0.5 in mRAS and cRAS, respectively. From Day 66–72 the Bray-Curtis similarity was 0.05 higher in mRAS than cRAS, again supporting that mRAS could be slightly more stable at the end of the experiment with increasing organic load than cRAS. A PERMANOVA test based on with Bray-Curtis similarities confirmed that the microbial community compositions from all samplings both within and between systems were significantly different ($p < .05$). Both systems underwent succession through the experiment, and the water microbiota changed slightly more in cRAS than in mRAS (lower Bray-Curtis similarities for between-day comparisons).

3.5. Microbial community compositions in mRAS and cRAS

The taxa summary (Fig. 7) shows the development of the microbial community composition at the class level. The data are presented as relative abundances where the shown taxa are the relative share (values from 0 to 1) of reads in the sample. The most abundant bacterial classes in the water were Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Actinobacteria. During P1 with increasing organic load in the systems, the relative abundance of Betaproteobacteria increased from approximately 0.12 to 0.3 in both systems. The opposite was observed for Gammaproteobacteria, which decreased a factor 5 from 0.25–0.3 (Day 1) to 0.05–0.07 (Day 18). In cRAS there was a transitory peak in the abundance of Alphaproteobacteria from Day 18 to 39 from 0.25 to 0.4, but the abundance remained at 0.25 in mRAS. At the end of P2 (Day 50) cRAS had a relative abundance of 0.15 of Actinobacteria, whereas it was only 0.02 in mRAS.

SIMPER analysis based on Bray-Curtis similarities was performed to determine which OTUs that contributed most to the difference between systems and sampling date (samples from the same system and sampling date were grouped: Fig. 8). An OTU representing *Gemmobacter* (Alphaproteobacteria: OTU_196) was the dominating OTU in cRAS at Day 39, with a relative abundance of 0.4. This is the highest relative abundance of any OTU at one sampling date in the whole dataset, and it contributed most to the difference between systems and sampling dates (6.3%). The relative abundance in mRAS was considerably lower (0.1) at Day 39, and in both systems the relative abundance dropped to less than 0.01 by Day 50. OTU_4 (*Sphaerotilus*, Betaproteobacteria) was more relative abundant in mRAS in P2 and P3 (0.2 and 0.1) than in

cRAS (0.1 and 0.05). OTU_5 (*Legionella*, Gammaproteobacteria) was highly abundant only in the beginning of the experiment at Day 1, with relative abundance of 0.27 in cRAS and 0.20 in mRAS. After Day 1 the relative abundance dropped to less than 0.01 and remained low through the rest of the experiment. The OTU table contained 57 different OTUs that belonged to the *Legionella* genus, and those OTUs were more abundant during P2 and P3 as well (Supplementary Fig. S1). An OTU representing *Mycobacterium* (Actinobacteria: OTU_7) was highly relative abundant in cRAS water towards the end of the experiment (up to around 0.25), while it had a relative abundance of <0.03 in mRAS through the whole experiment. Another group of bacteria that have been related to high particle environments in RAS which can create anaerobic zones, are sulphate reducing bacteria (SRB). In this study the relative abundance of SRBs (Supplementary Fig. S2) was higher in mRAS (0.12–0.16) than cRAS (0.01–0.06) in P2 and P3.

4. Discussion

4.1. Membrane filtration reduces the microbial CC by removal of small organic particles

The membrane treated a side stream of 10–15% of the total water flow, which significantly lowered organic carbon build-up (up to 50% lower) and consequently the density of bacteria in mRAS. This effect was especially strong when the organic loading was high. The effect of membrane filtration was evident for POC (Fig. 2A), but not for DOC (Fig. 2B). DOC is the most easily biologically degradable fraction of organic carbon, and 10–30% has typically been shown to be easily biologically degradable, depending on the water source (Søndergaard and Worm, 2001; Piech et al., 2019). In this experiment, organic matter was added to the systems every day through fish feed and production of faeces, and the reduction in POC through membrane filtration resulted in less solubilization and hydrolyzation of particles that eventually could become DOC. As more particles accumulated in cRAS, there was probably more DOC production in this system. The typically 2–6% higher DOC concentration in cRAS than in mRAS (Fig. 2B) indicate that the extra DOC produced was quickly consumed by the bacteria. In P2 the densities of bacteria decreased more rapidly after the bloom in mRAS than in cRAS (Fig. 3). In mRAS the membrane removed both bacteria and the bacterial substrate. In cRAS the bacteria and bacterial substrate were primarily removed through dilution due to water exchange. Bacteria can grow faster than the systems' HRTs (Since the HRTs were in the order of days: Table 2), causing the bacterial densities to decrease much slower in cRAS. The membrane showed a stabilizing effect on the bacterial CC during the experiment when the loading of organic matter changed. Such stabilization is thought to be beneficial for commercial fish production in RAS, which are subject to changes in organic loading due to introduction of new fish groups, weighing, vaccination, change of feed etc. through the production cycle. The level of the removal of organic matter is easy to control by deciding the size of the side stream that goes through the membrane, and one can thus avoid lowering the CC below a critical level (Holan et al., 2013; Vadstein et al., 2018b).

4.2. Membrane filtration in RAS increase the diversity of the microbial communities

The water microbiota of mRAS developed and maintained its higher diversity (i.e. richness and evenness: Fig. 5A and B), whereas cRAS microbiota developed into communities with lower diversity. It was the abundance of the different OTUs present in each RAS that changed the most, rather than the contribution from new OTUs throughout the experiment (Fig. 6B and C). Both systems were initially colonized by bacteria from the same fish and inlet water, and 70–80% of all OTUs identified were found in all samples between and within the systems. This shows that both RAS had a similar microbiome with respect to

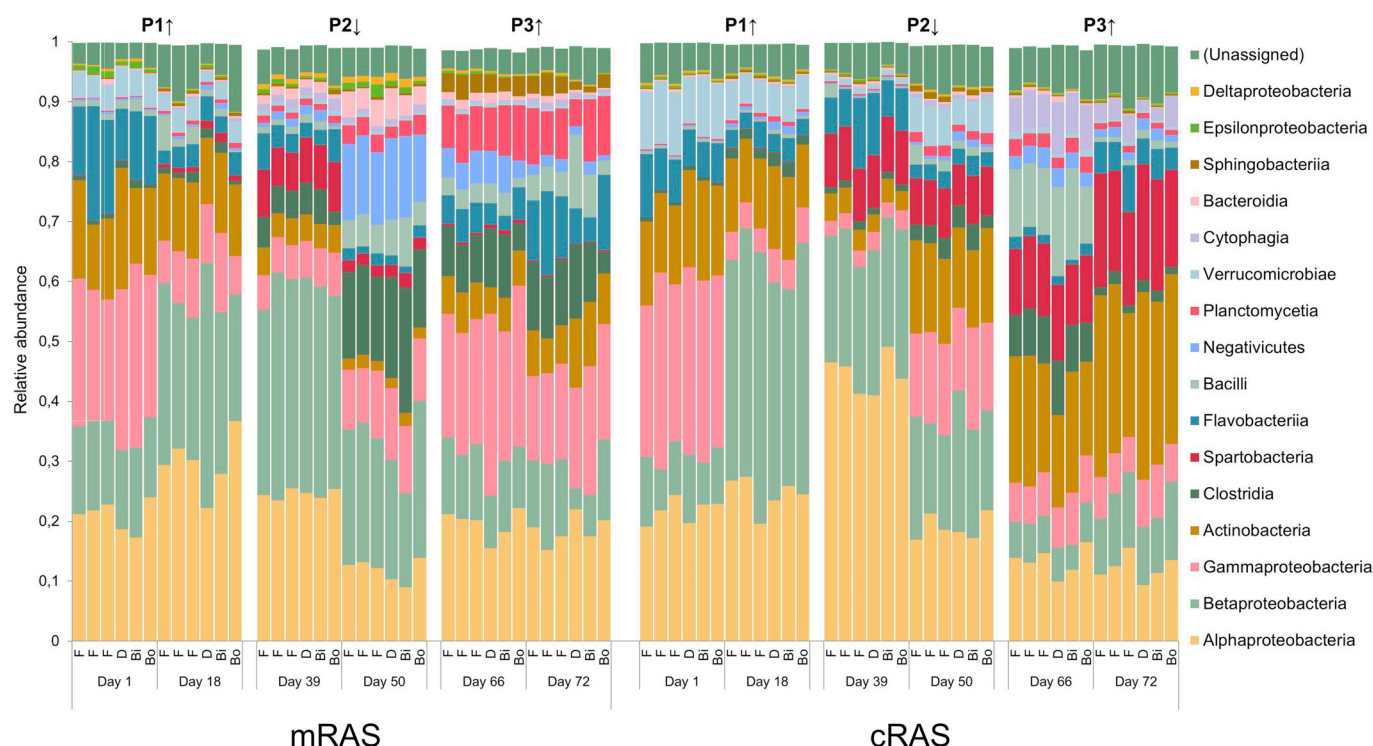


Fig. 7. Composition of water microbiota at class level showing relative abundance at the different sampling dates in mRAS and cRAS. Only taxa with >0.01 relative abundance in minimum one sample is included in this fig. F = fish tanks, D = drum screen filter, Bi = biofilter in, Bo = biofilter out.

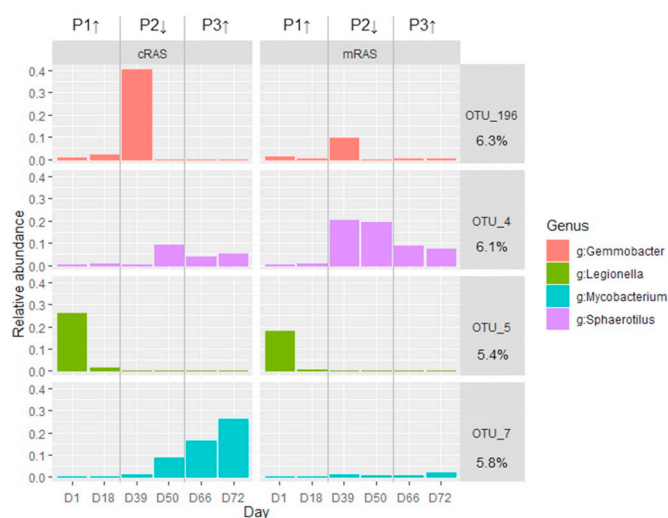


Fig. 8. Top four OTUs contributing to the difference between sampling dates and systems. All OTUs are classified for the genus (different colors) with a confidence threshold of 0.8. Relative abundance on y-axis and contribution to difference (%) from SIMPER analysis is shown below OTU IDs.

OTUs present, but that the differences in organic loading in each RAS created a selection pressure where the different bacteria succeeded to different degrees. When the organic matter loadings changed (between periods), the change in the microbial CC was not as profound in mRAS as in cRAS. This temporal destabilization in the environment caused the diversity to drop and specific bacteria became dominant, especially in cRAS, likely due to higher substrate availability. One example is OTU_196 (*Gemmobacter*, Alphaproteobacteria), which was four times more relative abundant in cRAS than mRAS on Day 39 (Fig. 8). Day 39 was approximately two weeks following the peak bacterial bloom and the organic load was decreasing. OTU_196 was not identical to any of the 16S type strains for *Gemmobacter* in the RDP database. The highest

match of 97.9% was with *Gemmobacter tilapia* (Sheu et al., 2013), a strain isolated from a freshwater pond with *Tilapia fish* (*Tilapia reindalli*). Sheu et al., 2013 did not report any disease of the fish and little information can be found about this genus. OTU_7 in the *Mycobacterium* genus was found in high relative abundance in cRAS. This genus includes several human pathogens (Gupta et al., 2018), and the SeqMatch tool showed that OTU_7 was not identical to any of the 16S type strains. The known species *Mycobacterium salmoniphilum* which cause mycobacteriosis in salmon farming (Aro et al., 2014) was not found to match the OTU_7 representative sequence. There were 57 different OTUs that belonged to the genus of *Legionella* (Gammaproteobacteria), and this genus was highly abundant throughout the experiment in both systems (Supplementary Fig. S1). *Legionella* is common in freshwater and soil environments, and around 50% of the species have been associated with disease in humans (Llewellyn et al., 2017). OTU_5 (Fig. 8) and the other most abundant *Legionella* OTUs (Supplementary Fig. S1) representative sequences had generally low sequence match to the different *Legionella* species in the RDP database, and *Legionella* is not known for causing losses in salmon farming. Amoeba are known to be hosts for *Legionella* as these bacteria commonly replicate intracellularly in eukaryotic hosts (Thomas et al., 2004). Thus, this high abundance of *Legionella* throughout the experiment could indicate high abundance of amoeba. Protozoan organisms were not targeted in this study, it could be interesting to focus more on the protozoa in RAS in the future. Both *Mycobacterium* and *Legionella* are commonly found in low amounts in municipal drinking water distribution systems (Waak et al., 2018; Waak et al., 2019), and the intake water for both RAS systems was municipal drinking water. The high relative abundance of *Mycobacterium* and *Legionella* in this study is probably due to the environment with high concentrations of nutrients. SRBs have received much attention recently in the commercial salmon RAS industry in Norway, as there have been reported several incidents with high salmon mortalities where production of toxic hydrogen sulphide (H_2S) is thought to have been the cause (Åtland and Stenberg, 2019). SRBs are part of the natural microbiota and are omnipresent (Vigneron et al., 2018). The relative abundance of SRBs was higher in mRAS (Supplementary Fig S2), even

though it had lower turbidity and POC than cRAS. It could be that in cRAS the higher concentrations of organic carbon caused methanogenic bacteria to compete with the SRBs in anaerobic spots (Tal et al., 2009). However, measurements to investigate this were not undertaken.

4.3. Did membrane filtration result in better water quality and performance of fish?

The membrane filtration resulted in better physicochemical water quality in terms of less particles/lower turbidity, less accumulation of organic carbon and slightly lower concentrations of TAN (Table 3). Both systems were RAS, which are considered to select for a more beneficial microbial water quality compared to traditional flow through systems (Attramadal et al., 2014; Vestrum et al., 2018). The concentrations of TOC and DOC were not noticeably higher than concentrations found in other RAS, ranging from 10 to 25 mg C L⁻¹ (Krumins et al., 2001; Hambly et al., 2015). Known salmon pathogenic genera were not found in high relative abundance in either of the systems and were not studied further. However, mRAS had significantly lower bacterial densities and higher bacterial diversity. This could indicate that mRAS developed a better microbial water quality than cRAS by providing more stable conditions for K-selection, namely lower and more stable concentrations of available organic matter, supporting the hypothesis for the experiment. Mature microbial communities dominated by K-strategists are predicted to have higher stability to perturbations (De Schryver and Vadstein, 2014; Vadstein et al., 2018b), which was more seen in mRAS than in cRAS. This further supports the hypothesis that a more microbially matured water can be achieved in RAS for juvenile salmon with appropriate use of membrane filtration. Membrane filtration will reduce the probability for microbially related accidents such as blooms of pathogens and potential anaerobic conditions that can lead to production of H₂S.

The average weight of the fish at the end of the experiment in mRAS was 14% and significantly higher than in cRAS, and mRAS produced more biomass in total. The better growth in mRAS can be attributed to a combination of higher temperature and better conditions. On average the temperature was 1.2 °C higher in mRAS than in cRAS (Table 3) caused by the membrane operation. Even though the temperature range in this experiment has shown little effect on the growth of salmon (Handeland et al., 2008), it is hard to conclude exactly how much of the improved growth of the fish that can be attributed to the higher temperature compared to the better water quality. Nevertheless, both the better water quality and the higher temperature should be weighed as a positive effect of the membrane, as heating of water is considered to be a large energy cost in RAS (Badiola et al., 2017). The estimated energy operating expenses (OPEX) for salmonids in RAS varies from 5.46 to 26 kWh kg⁻¹ of fish produced (Ayer and Tyedmers, 2009; d'Orbcastel et al., 2009; Summerfelt et al., 2009; Samuel-Fitwi et al., 2013; Liu et al., 2016; Song et al., 2019). The energy OPEX for membrane ultrafiltration is estimated to be 0.1–0.15 kWh m⁻³ of treated water (Pearce, 2008; Verrecht et al., 2010; Maere et al., 2011). Liu et al. (2016) reports a RAS OPEX cost of 5.4 kWh kg⁻¹, including the whole production cycle from hatching to market size salmon of 5 kg. Using the data provided by Liu et al. (2016) and adding membrane ultrafiltration (treating a side stream of 10% of the water flow) in all production stages, the energy OPEX of the membrane would equal 1.5 kWh kg⁻¹ fish, which equal 27% of the total energy OPEX cost of the RAS. Song et al. (2019) reports a cost of 7.5 kWh kg⁻¹ market size salmon produced. Estimating the cost of membrane filtration in that study, the membrane filtration OPEX constitutes 5.6% of the total RAS OPEX cost. Due to the high variability in estimated energy use in RAS, it is hard to conclude whether the cost of membrane filtration can be balanced by better water quality and fish growth. Moreover, inclusion of membrane filtration probably has a changing cost-benefit situation through the production cycle. It can for example, be more beneficial in stages and periods where the fish is more vulnerable to particles and the water

flow is relatively low, compared to production stages with more robust fish and high water flows. More research is therefore needed with membrane filtration in RAS at the different production stages of salmon to study the cost and the benefits. What we can conclude from our study is that the higher stability in physical, chemical and microbial water quality variables, indicate that the membrane had a stabilizing effect and reduce the carrying capacity for bacteria. If the RAS and fish had been challenged, we expect that the system with membrane filtration would have managed better. The better water quality and higher temperature led to better fish growth, and illustrates the potential of including membrane filtration in RAS for salmon smolt production.

5. Conclusions

- For microbial water quality, the system with membrane filtration had smaller and shorter bacterial blooms, generally lower densities of bacteria, and more diverse microbial communities. Microbially matured water was achieved in production of Atlantic Salmon parr in RAS, and the water seemed to be more mature with membrane filtration.
- For physicochemical water quality the system with membrane filtration had lower concentrations of particles/lower turbidity, less accumulation of organic carbon and slightly lower concentrations of TAN. In general, the variability of these measurements was lower in mRAS.
- The survival of the fish was the same in both systems, and a combination of better water quality and higher temperatures resulted in better growth of fish in mRAS than in cRAS.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2020.735268>.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The work was funded by the ERA-NET COFASP (Europe 2020 strategy), the Norwegian Research Council project “Water treatment technology for recirculating aquaculture systems to increase efficiency by reducing the negative effects of organic matter (RAS-ORGMAT) (260872/E40)”, NTNU Faculty of Engineering with Strategiske Omstillingsmidler (SO) and the NTNU Oceans pilot program on “Aquaculture & Environmental Interactions”. The authors wish to thank the staff at NTNU Sealab for their help during the experiment, especially Arne Kjosnes for all the technical and practical support.

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